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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/54, C12N 15/24, A61K 38/20 | A1

(11) International Publication Number: WO 96/09323

(43) International Publication Date: 28 March 1996 (28.03.96)

(21) International Application Number: PCT/EP95/03708

(22) International Filing Date: 20 September 1995 (20.09.95)

(30) Priority Data:
MI94A001916 21 September 1994 (21.09.94) IT

(71) Applicant (*for all designated States except US*): DOMPE' S.P.A [IT/IT]; Via Campo di Pile, I-67100 l'Aquila (IT).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): BORASCHI, Diana [IT/IT]; Via S. Martino, 12/12a, I-20122 Milano (IT). BOSSU', Paola [IT/IT]; Via S. Martino, 12/12a, I-20122 Milano (IT). RUGGIERO, Paolo [IT/IT]; Via S. Martino, 12/12a, I-20122 Milano (IT). MACCHIA, Giovanni [IT/IT]; Via S. Martino, 12/12a, I-20122 Milano (IT). TAGLIABUE, Aldo [IT/IT]; Via S. Martino, 12/12a, I-20122 Milano (IT). FRIGERIO, Francesco [IT/IT]; Via S. Martino, 12/12a, I-20122 Milano (IT). GRIFANTINI, Renata [IT/IT]; Piazza Garibaldi, 10, I-62032 Camerino (IT). FRASCOTTI, Gianni [IT/IT]; Via S. Martino, 12/12a, I-20122 Milano (IT). GRANDI, Guido [IT/IT]; Via S. Martino, 12/12a, I-20122 Milano (IT).

(74) Agent: MINOJA, Fabrizio; Studio Consulenza Brevettuale, Via Rossini, 8, I-20122 Milano (IT).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IL-1 RECEPTOR ANTAGONISTS WITH ENHANCED INHIBITORY ACTIVITY

(57) Abstract

DNA molecules that code for IL-1 antagonists with improved biological activity are described. DNA molecules coding for improved IL-1 antagonists inserted into expression vectors and host cells transformed with the said vectors containing the DNA coding for improved IL-1 antagonists and a method for the production of improved IL-1 antagonists in essentially pure form are also described. Preparations that can be injected or can be administered by some other route, consisting of a pharmaceutical preparation of the said mutants, are particularly useful as drugs in the field of therapy.

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IL-1 RECEPTOR ANTAGONISTS WITH ENHANCED INHIBITORY ACTIVITY

The present invention relates to mutants of the IL-1 receptor antagonist (IL-1ra), with an improved inhibitory activity compared to the wild type antagonist, means and methods for their preparation, and their use in the therapeutic sector in all pathologies in which IL-1 is thought to be involved.

Background of the invention

IL-1 (interleukin-1) is the cytokine that the body produces, in response to infections, various kinds of attack or antigenic stimulation, to initiate a defence reaction of the inflammatory or immune type. IL-1 is a polypeptide of approx. 17.5 kDa in its mature form, produced mainly by the macrophages but also by epidermal, lymphoid, vascular and epithelial cells. IL-1 is one of the principal stimulating factors of both the inflammatory and immune responses and, in its circulating form, it is capable of acting as a hormone, inducing a broad spectrum of systemic changes at metabolic, neurological, haematological and endocrinological level. Thus, IL-1 exerts an influence on mesenchymal tissue remodelling, contributing both to destructive processes and to repair processes. Furthermore, IL-1 is an activator of lymphocytes and plays a fundamental role in the initiation and amplification of the immune response. IL-1 also possesses strong activity of the inflammatory type, for example stimulation of the production of prostanoids and of proteases in various cells, including chondrocytes,

fibroblasts, synovial cells, and brain cells. Thus, IL-1 is involved in many components of the acute-phase response and is the endogenous mediator of fever (endogenous pyrogen). IL-1 can act in synergy with other cytokines, especially TNFα, significantly amplifying its inflammatory activity.

Cloning of IL-1 has led to the identification of two active forms. The predominant form is IL-1β, synthesized as an inactive precursor of 269 amino acids (31 kDa), which is then cut by a protease to give rise to the active mature form (corresponding to amino acids 117-269 of the precursor). A form that occurs about a hundred times less frequently, and is generally associated with the cells, is IL-1α, which has about 26% homology with IL-1β, and which is also synthesized as a precursor of 271 amino acids (which, however, possesses biological activity), which then gives rise to the mature form after proteolysis of the precursor. IL-1 represents a special case among the cytokines (together with fibrobalst growth factor = FGF) in that it lacks a signal peptide and so is not secreted via the normal routes. The most abundant extracellular form therefore consists of the mature form of IL-1β, which is thus responsible for the majority of the biological activities of IL-1, both immunostimulant and inflammatory.

In view of such activities, it has been hypothesized that IL-1 might have a role in the pathogenesis of inflammatory and autoimmune diseases. Thus, in the vast majority of pathologies of acute and chronic inflammation and in many autoimmune pathologies,

increased production of IL-1β has been identified as one of the main factors responsible for the pathology (Dinarello C.A. Blood 77: 1, 1991).

The biological activities of IL-1 are inhibited in the presence of specific inhibitors. In view of IL-1's fundamental role in the pathogenesis of many autoimmune diseases and of chronic inflammatory diseases with tissue destruction, it is suggested that inhibition of IL-1 could be useful in the treatment of these pathologies. IL-1ra (IL-1 receptor antagonist) is a cytokine that is structurally very similar to IL-1, but is synthesized with a signal peptide and secreted as mature glycosylated protein. A non-glycosylated intracellular form of IL-1ra, with seven extra amino acids and without a signal peptide, with activity comparable to that of secreted IL-1ra, has also been described. IL-1ra is capable of binding effectively to IL-$1R_I$ and much less well to IL-$1R_{II}$. IL-$1R_I$, the type I IL-1 receptor, is a receptor that belongs to the immunoglobulin superfamily, composed of an extracellular domain (which has three immunoglobulin-like units bound by disulphide bridges), a transmembrane sequence that anchors the receptor to the cell, and an intracellular domain that is responsible for transmitting the activation signal to the interior of the cell. The other IL-1 receptor, IL-$1R_{II}$, is structurally very similar to IL-$1R_I$ in the extracellular and transmembrane part, but possesses practically no intracellular domain and therefore does not seem capable of transmitting the activation signal. It is therefore hypothesized that IL-$1R_{II}$ does not have the ability to activate the cells and

that IL-$1R_I$ is largely responsible for cell activation in response to IL-1 (Arend W.P. J. Clin. Invest. 88: 1445, 1991; Dinarello C.A. & Thompson R.C. Immunol. Today 11: 404, 1991). IL-$1R_{II}$ is released naturally by the cell membrane, probably through the action of a specific protease, and once it is free in the extracellular space it is able to capture circulating IL-1β and prevent it from interacting with membrane IL-$1R_I$, so that it functions as an IL-1 inhibitor. However, the actual biological role of IL-$1R_{II}$, apart from capture and inhibition of IL-1 when released by the cell in soluble form, has not yet been elucidated definitively and there are data in various systems that suggest possible cell activation that is dependent on IL-$1R_{II}$ (Boraschi et al. Neuro-Immunology of Fever p. 19, 1992; Luheshi G. et al. Am. J. Physiol. 265: E585, 1993; Kent S. et al. Proc. Natl. Acad. Sci. USA 89: 9117, 1992). IL-1ra does not have IL-1-like biological activity, in that it occupies IL-$1R_I$ without activating the cell, and in occupying the receptor it functions as an antagonist of IL-1 activity. On account of its antagonist activity, IL-1ra has been used successfully in experimental models of inflammation induced by IL-1, by LPS (a bacterial endotoxin) and by live bacteria, to inhibit the inflammatory, toxic and lethal pathologic effects of the treatments (Ohlsson K. et al. Nature 348: 550, 1990; Wakabayashi G. et al. FASEB J. 5: 338, 1991; Alexander H.R. et al. J. Exp. Med. 173: 1029, 1991; Fischer E. et al. J. Clin. Invest. 89: 1551, 1992). However, in view of the extreme potency of IL-1 (which can activate cells by occupying fewer than ten

receptors/cell), the doses of IL-1ra necessary to obtain significant therapeutic effects in vivo are extremely high. Trials in humans in septic shock have shown a marginal efficacy of IL-1ra even at extremely high doses (Fischer C.J. et al., J.A.M.A. 271: 1836, 1994). Accordingly, it is particularly useful to be able to modify the structure of IL-1ra so as to increase its capacity for interaction with IL-$1R_I$ and so improve its therapeutic efficacy. An IL-1ra mutant having a glycine instead of asparagine in position 91 has been recently disclosed (Evans R. et al. 270, 11477, 1995).

Field of the invention

The present invention relates to IL-1ra mutants that can be used as drugs in the therapeutic field, to inhibit the pathogenetic activities of IL-1 with increased efficacy. More particularly, the field of the invention comprises:

- mutants of IL-1ra with improved inhibitory activity, characterized in that at least one of the two amino acid residues in positions 91 and 109 of the sequence of wild type (wt) IL-1ra is replaced by a different residue;
- DNA sequences coding for the said mutants;
- expression vectors comprising the said sequences;
- host microorganisms transformed with the said vectors;
- methods for the production of these mutants by culturing the transformed host microorganisms in appropriate conditions;
- use of the mutants of IL-1ra for inhibiting the biological activities of IL-1;

- pharmaceutical compositions comprising a therapeutically effective amount of at least one mutant of IL-1ra, a carrier and/or a pharmacologically acceptable solvent, which can be used for inhibiting the activities of IL-1, especially in situations where IL-1 could be involved in the pathological process.

Detailed description of the invention

The present invention relates to IL-1ra mutants and to pharmaceutical preparations containing them, as active principle for the therapeutic use as an IL-1 antagonist to inhibit the activities of IL-1 in vivo. The mutants of the invention have potential uses in the treatment of tumours, inflammatory and autoimmune diseases of the lung and airways, CNS, kidney, joints, endocardium, pericardium, eyes, ears, skin, gastrointestinal tract, urogenital system, in septic shock, bone and cartilage resorption, rheumatoid arthritis, atherosclerosis and other chronic inflammatory pathologies with or without autoimmune involvement.

The mutants of the invention are characterized by the replacement of the amino acid residue in position 91 with an amino acid residue selected from glutamine, arginine, lysine, histidine and tyrosine and/or in position 109 with an amino acid residue selected from serine, alanine, phenylalanine, valine, leucine, isoleucine, methionine. Said mutants are prepared by means of molecular techniques of mutagenesis, to produce polypeptides that contain the sequence of IL-1ra, modified so as to improve its capacity for interacting

with IL-$1R_I$ and therefore enhance its capacity as IL-1 antagonist. Table 1 shows the nucleotide and amino acid sequence of IL-1ra (Sequence Id N° 1 and 2, respectively). Table 2 shows the amino acid sequence of IL-1ra with indication of the selected mutations.

According to the present invention, after transformation of host microorganisms with expression vectors containing the sequences coding for the said mutants and culturing the said organisms, polypeptides with improved IL-1ra activity are obtained and purified, replacing the residue in position 91 and/or the residue in position 109 in the sequence of extracellular IL-1ra or of other members of the IL-1ra family by the above mentioned amino acid.

Preferably, the residue in position 91 is replaced by an arginine residue and the residue in position 109 by an alanine residue. A particularly preferred mutant has an arginine residue in position 91 and an alanine residue in position 109.

Example 2 describes the preparation of the improved mutants of IL-1ra according to the invention, denoted for conciseness by the following symbols:

MILRA-1 N91->R

MILRA-2 T109->A

MILRA-3 N91/T109->R/A

These improved mutants possess an enhanced capacity for binding to the type I IL-1 receptor (IL-$1R_I$), together with an enhanced capacity for binding to the type II IL-1 receptor (IL-$1R_{II}$), so as to provide increased efficacy of inhibition even in pathological situations where IL-1ra functions little owing to the

possible functional involvement of $IL\text{-}1R_{II}$ (neutrophilia, bone resorption, CNS effects, etc.).

To construct improved mutants of IL-1ra, the cDNA of IL-1ra was cloned in a suitable vector that would permit the appropriate genetic manipulations. The nucleotide sequence coding for IL-1ra is shown in Table 1 (Sequence Id N° 1), including the sequence that codes for the signal peptide of 25 amino acids. The amino acid sequence of IL-1ra is shown in Tables 1 and 2 (Sequence Id N° 2). Natural IL-1ra is expressed as a protein of 177 amino acids which, after removal of the signal sequence, gives rise to a mature molecule of 152 amino acids. The cDNA of IL-1ra is used for production of the IL-1ra protein, employing standard techniques. The gene can be inserted into an expression vector and the expression vector can be used for transforming a suitable host. The transformed host can be cultured in conditions that favour expression of the IL-1ra gene and, consequently, production of the IL-1ra protein.

These substitutions at positions 91 and/or 109 can be effected by site-specific mutagenesis using appropriate synthetic oligonucleotides. The recombinant gene is then inserted into the expression vector to produce the improved mutant.

For the therapeutic uses mentioned above, the mutated proteins of the invention will be administered in the form of pharmaceutical compositions suitable for parenteral, oral or topical administration, as described for example in "Remington's Pharmaceutical Sciences Handbook", Mack, Pub. Co., NY, USA, 17th ed. The average doses can vary from 2 μg/kg/h to 2 mg/kg/h by

intravenous infusion, and from 2 µg/kg/day to 2 mg/kg/day by other administration routes.

EXAMPLE 1a Cloning of the IL-1ra gene in E. coli

The cDNA coding for the IL-1ra protein is for example isolated by PCR from a cDNA pool prepared by conventional techniques from cells of monocyte-macrophage origin. The oligonucleotides that can be used for selective amplification of the cDNA coding for IL-1ra are shown:

IL-1ra forward: 5'--GATCATATGCGACCCTCTGGGAGAAAATCC--3'
(Sequence Id N° 3) NdeI

IL-1ra reverse: 5'--GATCTGCAGCTACTCGTCCTCCTGGAAG--3'
(Sequence Id N° 4) PstI

The forward oligonucleotide is designed so as to insert the NdeI restriction site immediately upstream from the codon encoding the first amino acid of the mature form of the protein (R26). The NdeI site permits insertion of a non-natural methionine which will constitute the initial amino acid of the recombinant proteins in question. Similarly, by means of the reverse oligonucleotide, a PstI restriction site is inserted immediately downstream from the stop codon of the protein. The amplified fragment is cloned at the NdeI and PstI sites of the expression vector pRSETA, obtaining the plasmid pRSETA-IL1ra. The map of the plasmid pRSETA-IL1ra is shown in Fig. 1.

EXAMPLE 1b Cloning of the IL-1ra gene in B. subtilis

The cDNA coding for the IL-1ra protein is for example isolated by PCR from a cDNA pool, prepared by conventional techniques from cells of monocyte-macrophage origin. The oligonucleotides that can be used

for selective amplification of the cDNA encoding IL-1ra are shown:

IL-1ra forward:

(Sequence Id N° 5)

5'-GG<u>GAATTC</u>TTATGCGACCCTCTGGGAGAAAATCC-3'

EcoRI

IL-1ra reverse: 5'--GG<u>CTGCAG</u>CTACTCGTCCTCCTGGAAG--3'

(Sequence Id N° 6) PstI

The forward oligonucleotide has been designed so as to insert the EcoRI restriction site immediately upstream from the codon encoding the amino acid methionine (indispensable for directing the start of translation of mRNA), followed by the codon encoding the first amino acid of the mature form of the protein (R26).

Similarly, by means of the reverse oligonucleotide, a PstI restriction site is inserted immediately downstream from the stop codon of the protein.

The amplified fragment is cloned at the EcoRI and PstI sites of the expression vector pSM671, obtaining the plasmid pSM441. The map of plasmid pSM671 is shown in Fig. 2.

EXAMPLE 2 <u>Mutations of the IL-1ra gene</u>

To obtain the desired mutations, part of the sequence coding for IL-1ra (amino acids 30-152) is transferred by cloning from plasmid pSM441 into the mutagenesis plasmid Bluescript SK$^+$ between the SpeI and PstI restriction sites, obtaining the plasmid BSK-IL1ra. Mutagenesis is effected using synthetic oligonucleotides, obtained with an Applied Biosystems 392 oligonucleotide synthesizer and utilizing phosphor-

amidite chemistry.

To obtain mutagenesis at site 91 of IL-1ra, the following complementary oligonucleotide, which is the reverse of the coding sequence, was used (Sequence Id N° 7):

1. 5' GTC CTG CTT TCT GCG CTC GCT CAG 3'
91

Sequence 1 can be modified on the anticodon corresponding to amino acid 91 so as to code for the other amino acids in that position.

To obtain the plasmid BSK-MILRA-1 (containing the mutation in position 91), the synthetic oligonucleotide is mixed with the single strand of DNA from the plasmid BSK-IL-1ra in a pairing buffer (5 pmol of oligonucleotide with 0.2 pmol of single strand in 10 ml of buffer), the mixture is heated to 70°C, then cooled slowly to 30°C in 40 min and finally placed in ice. 1 ml of synthesis buffer, 1 ml (3 units) of T4 DNA ligase and 1 ml (0.5 unit) of T7 DNA polymerase are added to the mixture. After incubation for 1 hour at 37°C, the mixture is used to transform the competent cells. Identification of the positive clones is carried out by DNA sequencing.

Similarly, to obtain the plasmid BSK-MILRA-2, containing the T109->A mutation, the following oligonucleotide is synthesized (Sequence Id N° 8):

2. 5' CTC AAA ACT GGC GGT GGG GCC 3'
109

Alternatively, it is possible to use suitable oligonucleotides that code for the other amino acids in position 109. Then the same procedure is followed as for the N91->R mutation.

EXAMPLE 3 Insertion of the modified IL-1ra genes into expression vectors

The mutated sequence in plasmid BSK-MILRA-1 or BSK-MILRA-2 (mutation in position 91 or 109) is cut with SpeI and PstI and cloned directly into the expression plasmid pRSETA-IL1ra, between the same restriction sites, obtaining the expression plasmids pT7MILRA-1 and pT7MILRA-2. Cloning of the mutated sequences into the expression vector pSM441 was accomplished similarly, obtaining the expression plasmids pSM539 and pSM540. The sequence of the double mutant N91->R and T109->A is obtained by cloning between the SpeI and PstI sites of the vector pRSETA-IL1ra or of the vector pSM441, the SpeI-HaeII fragment of the BSK-MILRA-1 clone (amino acids 30-96) and HaeII-PstI fragment of the BSK-MILRA-2 clone (amino acids 97-152), obtaining the clone pT7MILRA-3 and pSMILRA3 respectively.

EXAMPLE 4 Expression of the modified genes of IL-1ra

The expression plasmids pT7MILRA-1, pT7MILRA-2 and pT7MILRA-3 are transferred independently into cells of E. coli strain BL21 (DE3), which possess the gene for T7 RNA polymerase and so are capable of transcribing coding sequences downstream from plasmid T7. The cells are grown in LB culture medium containing 100 mg/l of ampicillin until an OD_{590nm} of 0.7 is reached. Expression is induced at this point with 0.4 mM IPTG for 3-4 hours. The cells containing the protein expressed are harvested by centrifugation and frozen at -80°C until the time of purification.

The expression plasmids pSM539, pSM540 and pSMILRA3 are transferred independently to cells of B. subtilis

strain SMS118. The cells are grown in LB culture medium containing 5 mg/l of chloramphenicol for 16 hours at 30-37°C. The cells containing the protein expressed are harvested by centrifugation and frozen at -80°C until the moment of purification.

EXAMPLE 5 Purification of the modified proteins expressed

For the mutant proteins of IL-1ra with improved activity to be obtained in an essentially pure form, extraction from the bacteria and purification of the homogenate are undertaken, for example according to the procedures indicated below.

1. Extraction: The bacteria are thawed, resuspended 1:3 (wet weight:volume) in 25 mM MES pH 6.25, 1 mM EDTA (buffer A) and sonicated in melting ice for 5 min (E. coli) or 15 min (B. subtilis) at a power of 60-70 W at intervals of 30 s. The homogenate is centrifuged at 30 000 x *g* at 4°C (or is put through some other suitable operation, e.g. tangential filtration). Aliquots of the supernatant and sediment are analysed in SDS-PAGE. Alternatively, extraction from the bacteria can be effected by some other suitable method. Aliquots of the supernatant and of the sediment are analysed in SDS-PAGE.

2. Q-Sepharose FF: The supernatant is adjusted to pH 6.0-6.5 and to a conductivity of 3-5 mS/cm and batch-incubated for 3 hours at 4°C with stirring, with 1 ml/g of cells (wet weight) of Q-Sepharose Fast Flow (or some other suitable stationary ion-exchange phase) equilibrated in buffer A. Alternatively, the treatment can be carried out in a column, with isocratic elution

in buffer A. The unadsorbed matter is then collected by filtration on a porous diaphragm. The gel is washed for 20 min as above in 2 volumes of buffer A; the wash liquid is collected as above. Aliquots of the unadsorbed matter and of the wash liquid are analysed in SDS-PAGE. The mutant protein of IL-1ra is found in the unadsorbed matter and in the wash liquid.

Point 2 can be postponed and effected in a column with isocratic elution in buffer A instead of gel filtration as in point 5.

3. S-Sepharose FF: The unadsorbed matter and the wash liquid of the Q-Sepharose FF are combined, filtered on 0.45 mm and loaded onto a column of S-Sepharose Fast Flow (or some other suitable stationary ion-exchange phase) equilibrated in buffer A. The unadsorbed matter is collected and buffer A is passed through until the baseline (A_{280}) falls to zero. Then a linear gradient is applied from 0.05 to 0.5 M NaCl in buffer A in 2 column volumes, collecting the fractions. Alternatively, the linear gradient can be replaced by a stepped gradient, with intervals of 0.1 M NaCl. Aliquots of the unadsorbed matter, of the fractions from gradient elution and of the eluted peak are analysed in SDS-PAGE. The mutant IL-1ra protein is found in the central fractions of the first peak eluted between 0.2 and 0.4 M NaCl. Passage through Q-Sepharose FF and through S-Sepharose FF, as in points 2 and 3, can be reversed.

4. Filtration: The eluted peak of IL-1ra is concentrated and dialysed on Millipore Centriprep 10 filters, until the NaCl concentration falls below 50 mM; high molecular weight contaminants are removed by

filtration on Millipore Centricon 100 centrifuge filters. Aliquots from the various filtrates are analysed in SDS-PAGE. Depending on the volumes to be treated, the operations described can be performed with various systems, using the same type of membrane.

5. Bio Gel P10: Any contaminants present, whether of higher or lower molecular weight, are removed by gel filtration in a column of Bio Gel P10 from Bio-Rad (or some other equivalent stationary phase for gel filtration) equilibrated and eluted in buffer A. Aliquots of the fractions eluted are analysed in SDS-PAGE. The mutant IL-1ra protein is found in the middle fractions of the first peak eluted after the excluded volume peak, in essentially pure form.

EXAMPLE 6 Characterization of inhibitory activity with the assay of binding to IL-1R_I and IL-1R_{II}

The inhibitory activity of the improved IL-1ra mutants is measured in receptor binding assays, using IL-1ra as the reference standard. Cells which express IL-1R_I selectively (e.g. the murine thymoma clone EL4-6.1) and cells which express IL-1R_{II} selectively (e.g. Burkitt's human lymphoma RAJI clone 1H7) are chosen as target cells. The number of receptors per cell and the binding affinity (Kd) in both the lines are calculated from saturation curves obtained by incubating the cells (10^6 cells/test tube in a final volume of 0.1 ml of culture medium with NaN_3) with increasing doses of IL-1β labelled with ^{125}I in the presence or absence of a 500-fold molar excess of unlabelled IL-1β (for calculating non-specific binding, generally always less than 5-10%) for the optimum times and at the

optimum temperatures for attaining equilibrium (generally 2 hours at room temperature) (Scapigliati et al. FEBS Lett. 243: 394, 1989). To calculate the inhibition activity, tests are conducted by incubating the cells with a concentration of radiolabelled IL-1β corresponding to approximately half of the Kd in the absence or in the presence of stepped doses of IL-1ra (reference standard) or of the improved mutant.

The improved inhibitory activity of the mutants is determined by calculating the shift of the inhibition curve towards the lower doses when compared with the curve of IL-1ra. An example of the results obtained (for MILRA-1) is given in Table 3.

EXAMPLE 7 Characterization of antagonist activity with in-vitro assays of inhibition of IL-1β

The antagonist activity of the improved mutants of IL-1ra is evaluated by means of in-vitro biological assays of IL-1 activity. Two assays of this kind are described below.

1. Proliferation of murine thymocytes: normal thymocytes are obtained by breaking up thymus glands of C3H/HeJ mice (resistant to bacterial endotoxin) aged 4-8 weeks. The thymocytes ($5x10^5$ cells/well of Cluster^{96} plates) are incubated for 72 hours in RPMI-1640 culture medium with addition of antibiotics, L-glutamine, 2-ME, HEPES and 5% foetal calf serum at 37°C in air with 5% CO_2. Incubation takes place in the presence of a suboptimal dose of the mitogen PHA (1.5 mg/ml, which does not induce significant proliferation of thymocytes) and stepped concentrations of IL-1β. To determine the proliferation of the thymocytes induced by IL-1, at the

end of 72 hours of incubation 25 ml of culture medium containing 0.5 mCi of tritiated thymidine are added to each well. After another 18 hours, the cells from each well are harvested onto small glass-fibre disks and the radioactivity incorporated (proportional to the proliferation of the cells) is measured with a β-counter. For determination of the inhibitory capacity of the improved mutants of IL-1ra, the proliferation of the thymocytes is measured in response to the minimum dose of IL-1 that induces optimum proliferation (generally around 0.3 ng/ml) in the absence or in the presence of stepped doses of IL-1ra (control standard) or of the improved mutants. The improved inhibitory activity of the mutants is determined by calculating the shift of the inhibition curve towards the lower doses when compared with the curve of IL-1ra. An example of the results obtained (for MILRA-1) is presented in Table 3.

2. Induction of IL-6: cells of the continuous line of human osteosarcoma MG-63 are incubated (5×10^4 cells/well of Cluster^{96}) for 48 hours in RPMI-1640 culture medium with antibiotics, L-glutamine, HEPES and 5% foetal calf serum in the absence or in the presence of stepped doses of IL-1β.

The quantity of IL-6 in the culture supernatants is measured by a commercial ELISA assay or determined in the biological assay of proliferation of 7TD1 cells. For evaluation of the antagonist capacity of the improved mutants of IL-1ra, the production of IL-6 is measured in MG-63 cells stimulated with the minimum dose of IL-1 capable of optimum induction of IL-6 (around 0.3 ng/ml) in the absence or in the presence of stepped doses of

IL-1ra (control standard) or of the improved mutants of IL-1ra. The improved inhibitory activity of the mutants is determined by calculating the shift of the inhibition curve towards the lower doses when compared with the curve of IL-1ra.

An example of the results obtained (for MILRA-1) is presented in Table 3.

EXAMPLE 8 <u>Characterization of antagonist activity by in-vivo assays of inhibition of IL-1β</u>

The inhibitory activity of the improved mutants of IL-1ra is evaluated using in-vivo biological assays of IL-1 activity. Two assays of this kind are described below.

1. Induction of hypoglycaemia: C3H/HeJ mice or mice of some other strain (3-5 mice/test group) receive stepped doses of IL-1β by intraperitoneal administration. After two hours the animals are killed and the blood is collected for preparation of the serum. The serum glucose content is determined after reaction with glucose oxidase in a commercial colorimetric assay. To evaluate the antagonist capacity of the improved mutants of IL-1ra, induction of hypoglycaemia in vivo is effected by administering the minimum dose of IL-1β capable of inducing the optimum effect (generally around 5 mg/kg) in the absence or in the presence of stepped doses of IL-1ra (control standard) or of the improved mutants of IL-1ra.

The improved inhibitory activity of the mutants is determined by calculating the shift of the inhibition curve towards the lower doses when compared with the curve of IL-1ra. An example of the results obtained (for

MILRA-1) is presented in Table 3.

2. Induction of neutrophilia: C3H/HeJ mice or mice of some other strain (3 mice/test group) receive stepped doses of IL-1β with a single intraperitoneal administration. The increase of circulating neutrophils induced by IL-1 is evaluated by cytofluorimetry four hours after the treatment. The antagonist capacity of the improved mutants of IL-1ra is evaluated as inhibition of the neutrophilia induced by the minimum dose of IL-1 necessary to obtain the optimum increase of circulating neutrophils (generally around 150 ng/kg). IL-1ra (control standard) and the improved mutants are administered intraperitoneally on three occasions, at times -15 min, 0, +15 min, relative to IL-1β. The improved inhibitory activity of the mutants is determined by calculating the shift of the curve of inhibition towards the lower doses when compared with the curve of IL-1ra.

An example of the results obtained (for MILRA-1) is presented in Table 3.

TABLE 1

NUCLEOTIDE AND AMINO ACID SEQUENCE OF IL-1ra

```
1     ATGGAAATCTGCAGAGGCCTCCGCAGTCACCTAATCACTCTCCTCCTCTTCCTGTTCCAT
1     M  E  I  C  R  G  L  R  S  H  L  I  T  L  L  L  F  L  F  H

61    TCAGAGACGATCTGCCGACCCTCTGGGAGAAAATCCAGCAAGATGCAAGCCTTCAGAATC
21    S  E  T  I  C  R  P  S  G  R  K  S  S  K  M  Q  A  F  R  I

121   TGGGATGTTAACCAGAAGACCTTCTATCTGAGGAACAACCAACTAGTTGCTGGATACTTG
41    W  D  V  N  Q  K  T  F  Y  L  R  N  N  Q  L  V  A  G  Y  L

181   CAAGGACCAAATGTCAATTTAGAAGAAAAGATAGATGTGGTACCCATTGAGCCTCATGCT
61    Q  G  P  N  V  N  L  E  E  K  I  D  V  V  P  I  E  P  H  A

241   CTGTTCTTGGGAATCCATGGAGGGAAGATGTGCCTGTCCTGTGTCAAGTCTGGTGATGAG
81    L  F  L  G  I  H  G  G  K  M  C  L  S  C  V  K  S  G  D  E
```

- continued -

- continued -

```
301  ACCAGACTCCAGCTGGAGGCAGTTAACATCACTGACCTGAGCGAGAACAGAAAGCAGGAC
101  T  R  L  Q  L  E  A  V  N  I  T  D  L  S  E  N  R  K  Q  D

361  AAGCGCTTCGCCTTCATCCGCTCAGACAGTGGCCCCACCACCAGTTTTGAGTCTGCCGCC
121  K  R  F  A  F  I  R  S  D  S  G  P  T  T  S  F  E  S  A  A

421  TGCCCCGGTTGGTTCCTCTGCACAGCGATGGAAGCTGACCAGCCCGTCAGCCTCACCAAT
141  C  P  G  W  F  L  C  T  A  M  E  A  D  Q  P  V  S  L  T  N

481  ATGCCTGACGAAGGCGTCATGGTCACCAAATTCTACTTCCAGGAGGACGAG     531
161  M  P  D  E  G  V  M  V  T  K  F  Y  F  Q  E  D  E      177
```

TABLE 2

AMINO ACID SEQUENCE OF IL-1ra

					5
MEICR	*GLRSH*	*LITLL*	*LFLFH*	*SEITC*	RPSGR
10	15	20	25	30	35
KSSKM	QAFRI	WDVNQ	KTFYL	RNNQL	VAGYL
40	45	50	55	60	65
QGPNV	NLEEK	IDVVP	IEPHA	LFLGI	HGGKM
70	75	80	85	90	95
CLSCV	KSGDE	TRLQL	EAVNI	TDLSE	**N**RKQD
					R
100	105	110	115	120	125
KRFAF	IRSDS	GPT**T**S	FESAA	CPGWF	LCTAM
		A			
130	135	140	145	150	152
EADQP	VSLTN	MPDEG	VMVTK	FYFQE	DE

NOTES: The amino acids in italics represent the signal peptide, which is removed in the mature protein.

The numbering refers to the mature protein, without the signal peptide.

The amino acids in bold represent the two positions where the substitutions were planned, with the preferred substitution shown below.

TABLE 3

BIOLOGICAL ACTIVITY OF MILRA-1, MILRA-2 AND MILRA-3

BIOLOGICAL ASSAY OF IL-1β INHIBITION	IL-1ra	MILRA-1	MILRA-2	MILRA-3
In vitro:				
Binding to $IL\text{-}1R_I$*	0.34 nM	0.17 nM	0.19 nM	0.22 nM
Binding to $IL\text{-}1R_{II}$*	19.60 nM	13.20 nM	10.80 nM	70.70 nM
Thymocyte proliferation**				
to IL-1β 300 pg/ml	4.10 ng/ml	1.78 ng/ml	1.75 ng/ml	n.t.
to IL-1β 30 pg/ml	813 pg/ml	n.t.	n.t.	285 pg/ml
IL-6 production**	3.60 ng/ml	0.62 ng/ml	1.12 ng/ml	n.t.
In vivo:				
Hypoglycaemia***	968.0 µg/kg	128.0 µg/kg	n.t.	76.0 µg/kg
Neutrophilia****	225.0 µg/kg	<22.5 µg/kg	n.t.	n.t.

* Displacement of ^{125}I IL-1β equilibrium binding 80.4-0.6 Nm ^{125}I IL-1β on 10^6 cells).

** Murine thymocyte proliferation in response to 0.3 ng/ml of IL-1β.

*** Hypoglycaemia induced by in the mouse by 5.0 µg/kg IL-1β.

**** Neutrophilia induced in the mouse by 150 ng/kg of IL-1β.

Doses reported are ID_{50} except for neutrophilia, where ID_{100} is indicated.

n.t. not tested

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Dompe' s.p.a.
(B) STREET: via Campo di Pile
(C) CITY: L'Aquila
(E) COUNTRY: ITALY
(F) POSTAL CODE (ZIP): I-67100
(G) TELEPHONE: +39 862 3381
(H) TELEFAX: +39 862 338219

(ii) TITLE OF INVENTION: IL-1 receptor antagonists with increased inhibitory activity

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: IT MI 94/A 001916
(B) FILING DATE: 21-SEP-1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 531 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..531

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..75

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 76..531

(ix) FEATURE:
(A) NAME/KEY: mutation
(B) LOCATION: replace(346..348, "cgc")
(D) OTHER INFORMATION: /note= "CGC is the codon for the preferred

```
                Asn -> Arg aminoacid substitution at this
                position."

    (ix) FEATURE:
         (A) NAME/KEY: mutation
         (B) LOCATION: replace(400..402, "gcc")
         (D) OTHER INFORMATION: /note= "GCC is the codon for the preferred
                Thr -> Ala aminoacid substitution at this
                position."

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATG GAA ATC TGC AGA GGC CTC CGC AGT CAC CTA ATC ACT CTC CTC CTC       48
Met Glu Ile Cys Arg Gly Leu Arg Ser His Leu Ile Thr Leu Leu Leu
-25                 -20                 -15                 -10

TTC CTG TTC CAT TCA GAG ACG ATC TGC CGA CCC TCT GGG AGA AAA TCC       96
Phe Leu Phe His Ser Glu Thr Ile Cys Arg Pro Ser Gly Arg Lys Ser
                 -5                   1               5

AGC AAG ATG CAA GCC TTC AGA ATC TGG GAT GTT AAC CAG AAG ACC TTC      144
Ser Lys Met Gln Ala Phe Arg Ile Trp Asp Val Asn Gln Lys Thr Phe
         10                  15                  20

TAT CTG AGG AAC AAC CAA CTA GTT GCT GGA TAC TTG CAA GGA CCA AAT      192
Tyr Leu Arg Asn Asn Gln Leu Val Ala Gly Tyr Leu Gln Gly Pro Asn
     25                  30                  35

GTC AAT TTA GAA GAA AAG ATA GAT GTG GTA CCC ATT GAG CCT CAT GCT      240
Val Asn Leu Glu Glu Lys Ile Asp Val Val Pro Ile Glu Pro His Ala
 40                  45                  50                  55
```

```
CTG TTC TTG GGA ATC CAT GGA GGG AAG ATG TGC CTG TCC TGT GTC AAG        288
Leu Phe Leu Gly Ile His Gly Gly Lys Met Cys Leu Ser Cys Val Lys
                 60                  65                  70

TCT GGT GAT GAG ACC AGA CTC CAG CTG GAG GCA GTT AAC ATC ACT GAC        336
Ser Gly Asp Glu Thr Arg Leu Gln Leu Glu Ala Val Asn Ile Thr Asp
             75                  80                  85

CTG AGC GAG AAC AGA AAG CAG GAC AAG CGC TTC GCC TTC ATC CGC TCA        384
Leu Ser Glu Asn Arg Lys Gln Asp Lys Arg Phe Ala Phe Ile Arg Ser
         90                  95                 100

GAC AGT GGC CCC ACC ACC AGT TTT GAG TCT GCC GCC TGC CCC GGT TGG        432
Asp Ser Gly Pro Thr Thr Ser Phe Glu Ser Ala Ala Cys Pro Gly Trp
    105                 110                 115

TTC CTC TGC ACA GCG ATG GAA GCT GAC CAG CCC GTC AGC CTC ACC AAT        480
Phe Leu Cys Thr Ala Met Glu Ala Asp Gln Pro Val Ser Leu Thr Asn
120                 125                 130                 135

ATG CCT GAC GAA GGC GTC ATG GTC ACC AAA TTC TAC TTC CAG GAG GAC        528
Met Pro Asp Glu Gly Val Met Val Thr Lys Phe Tyr Phe Gln Glu Asp
                140                 145                 150

GAG                                                                    531
Glu
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 177 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Glu Ile Cys Arg Gly Leu Arg Ser His Leu Ile Thr Leu Leu Leu
-25                 -20                 -15                 -10

Phe Leu Phe His Ser Glu Thr Ile Cys Arg Pro Ser Gly Arg Lys Ser
                -5                   1               5

Ser Lys Met Gln Ala Phe Arg Ile Trp Asp Val Asn Gln Lys Thr Phe
         10                  15                  20

Tyr Leu Arg Asn Asn Gln Leu Val Ala Gly Tyr Leu Gln Gly Pro Asn
     25                  30                  35

Val Asn Leu Glu Glu Lys Ile Asp Val Val Pro Ile Glu Pro His Ala
 40                  45                  50                  55

Leu Phe Leu Gly Ile His Gly Gly Lys Met Cys Leu Ser Cys Val Lys
                60                  65                  70

Ser Gly Asp Glu Thr Arg Leu Gln Leu Glu Ala Val Asn Ile Thr Asp
             75                  80                  85

Leu Ser Glu Asn Arg Lys Gln Asp Lys Arg Phe Ala Phe Ile Arg Ser
         90                  95                 100
```

```
Asp Ser Gly Pro Thr Thr Ser Phe Glu Ser Ala Ala Cys Pro Gly Trp
    105                 110                 115

Phe Leu Cys Thr Ala Met Glu Ala Asp Gln Pro Val Ser Leu Thr Asn
120                 125                 130                 135

Met Pro Asp Glu Gly Val Met Val Thr Lys Phe Tyr Phe Gln Glu Asp
                140                 145                 150

Glu
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
- (A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
GATCATATGC GACCCTCTGG GAGAAAATCC                                    30
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GATCTGCAGC TACTCGTCCT CCTGGAAG 28

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GGGAATTCTT ATGCGACCCT CTGGGAGAAA ATCC 34

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GGCTGCAGCT ACTCGTCCTC CTGGAAG 27

```
(2) INFORMATION FOR SEQ ID NO: 7:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 24 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

  (iii) HYPOTHETICAL: NO

  (iii) ANTI-SENSE: NO

    (v) FRAGMENT TYPE: internal

   (vi) ORIGINAL SOURCE:
         (A) ORGANISM: Homo sapiens

   (ix) FEATURE:
         (A) NAME/KEY: -
         (B) LOCATION: 13..15
         (D) OTHER INFORMATION: /note= "anticodon corresponding to
              aminoacid 91 of mature IL-1ra"

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTCCTGCTTT CTGCGCTCGC TCAG                                      24
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: 10..12
(D) OTHER INFORMATION: /note= "anticodon corresponding to aminoacid 109 of mature IL-1ra"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CTCAAAACTG GCGGTGGGGC C 21

CLAIMS

1. Mutants of IL-1ra in which at least one of the amino acid residues in positions 91 and 109 of the sequence of wt IL-1ra is replaced by a residue selected from glutamine, arginine, lysine, histidine and tyrosine for position 91 and by a residue selected from serine, alanine, phenylalanine, valine, leucine, isoleucine and methionine for position 109.

2. Mutant of IL-1ra according to Claim 1, in which the amino acid residue in position 91 of the sequence of wt IL-1ra is replaced by arginine.

3. Mutant of IL-1ra according to Claim 1, in which the amino acid residue in position 109 of the sequence of wt IL-1ra is replaced by alanine.

4. Mutant of IL-1ra according to one of Claims 1-3, in which both of the amino acid residues in positions 91 and 109 of the sequence of wt IL-1ra are replaced respectively by arginine and alanine.

5. Mutants of IL-1ra according to one of Claims 1-4, in which the sequence of IL-1ra belongs to extracellular IL-1ra or to other members of the IL-1ra family.

6. DNA sequences coding for the mutants of IL-1ra of Claims 1-5.

7. DNA sequences coding for the mutants of IL-1ra according to Claim 6, also comprising regulating elements that permit their insertion into expression vectors.

8. Expression vectors containing a nucleotide sequence of Claim 6 or 7.

9. Host cells transformed with the expression vectors

as in Claim 8.

10. Method for the production, in essentially pure form, of the mutant proteins of IL-1ra of Claims 1-5 which comprises culturing the host cells of Claim 9 and recovering the expression product from the cells or from the culture medium.

11. Pharmaceutical compositions of Claims 1-6, containing as active principle a mutant of IL-1ra mixed with a suitable vehicle.

12. Use of the mutants of IL-1ra of Claims 1-6 for the preparation of a drug capable of antagonizing, in living organisms including man, the inflammatory, neurological, endocrinological, haematological, metabolic, catabolic and immunostimulant effects caused directly by IL-1 or caused by agents/pathologies in which endogenously produced IL-1 is involved in the pathology.

13. Use of the mutants of IL-1ra of Claims 1-6 for the preparation of a drug that can be used for the treatment of acute or chronic inflammatory pathologies, pathological disfunctions of the immune system, such as solid tumours and leukaemias, rheumatoid arthritis and arthralgias, autoimmune diseases, hyperreactivity and allergies, graft rejection, acute and chronic inflammatory diseases and autoimmune diseases of the lung and of the airways, of the CNS, of the kidney and of the urogenital system, of the skin, of the gastrointestinal tract, of the joints, of the eye, of the ear, of the pericardium and of the endocardium, endotoxic shock and septic shock by Gram-negatives and by Gram-positives, infections, bone and cartilage resorption, osteoporosis, atherosclerosis and other

pathologies of chronic inflammation with or without autoimmune involvement.

Fig. 1

MAP OF THE EXPRESSION PLASMID pRSETA-IL1ra

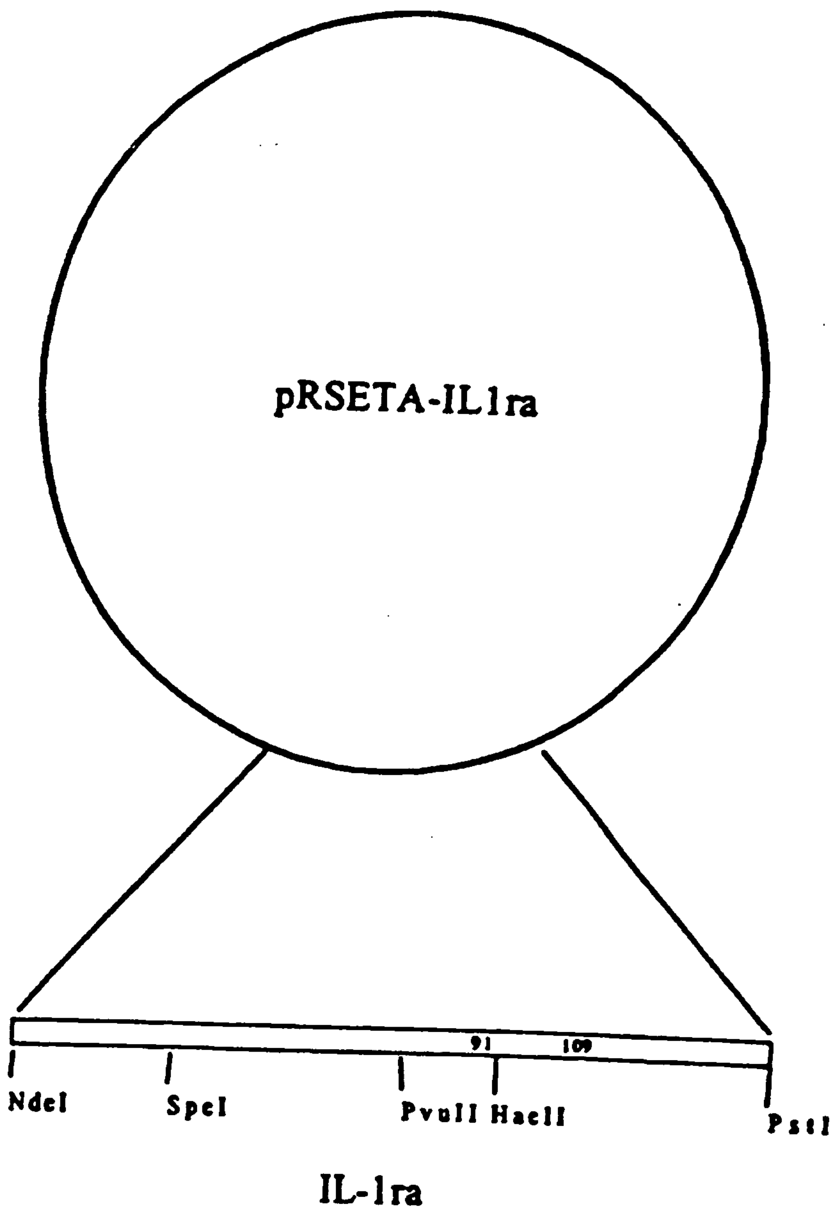
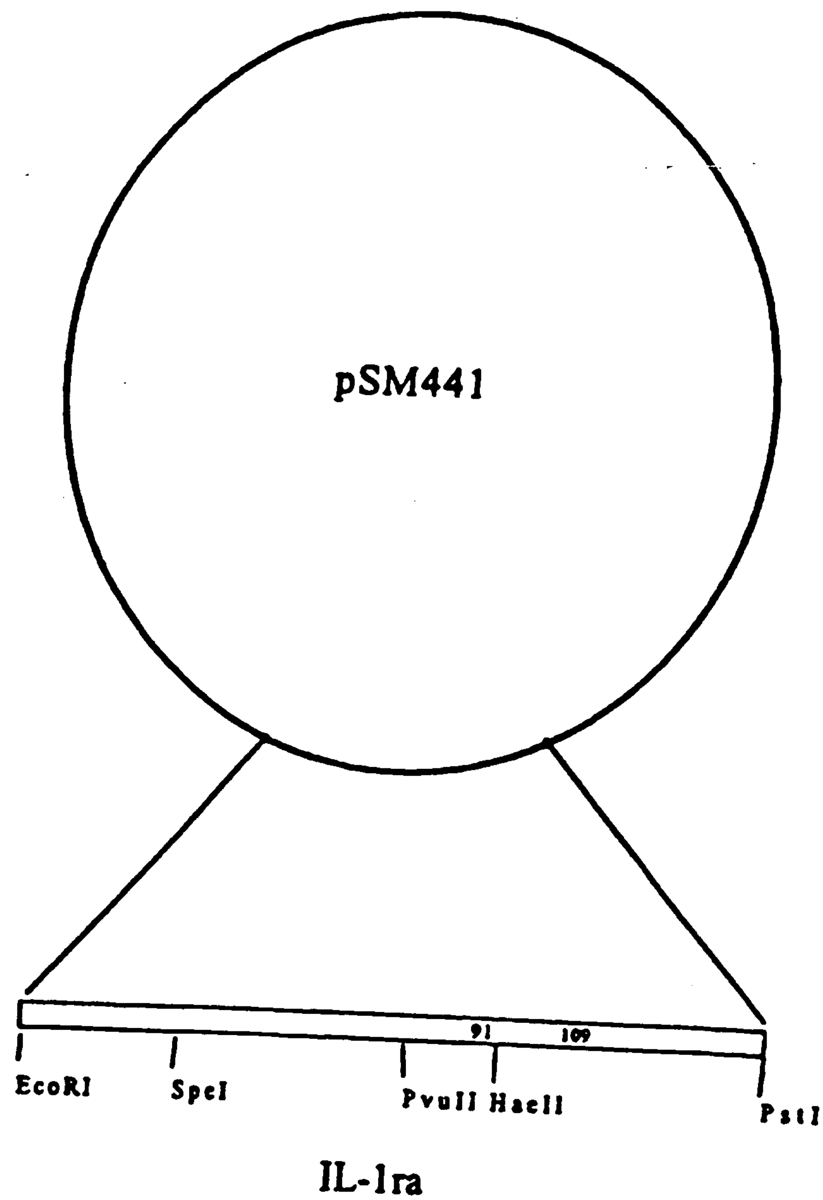


Fig. 2

MAP OF THE EXPRESSION PLASMID pSM441



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP 95/03708

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/54 C12N15/24 A61K38/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 88, April 1991 WASHINGTON US, pages 2658-2662, G. JU ET AL. 'CONVERSION OF THE INTERLEUKIN 1 RECEPTOR ANTAGONIST INTO AN AGONIST BY SITE SPECIFIC MUTAGENESIS.' see the whole document ---	1-13
A	WO,A,92 16221 (SYNERGEN, INC.) 1 October 1992 see claims 47-50; examples V,VI ---	1-13

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 February 1996	29.02.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ryckebosch, A

International Application No
PCT/EP 95/03708

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	INTERNATIONAL JOURNAL OF IMMUNOPATHOLOGY AND PHARMACOLOGY, vol. 7, no. 3, pages 235-239, A. TAGLIABUE ET AL. 'PHARMACOLOGICAL EXPLOITATION OF IL-1 AND IL-1 RELATED MOLECULES.' SEPTEMBER-DECEMBER 1994 see page 235, paragraph 2 - page 236, paragraph 1 ---	1-13
P,A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 19, 12 May 1995 MD US, pages 11477-11483, R.J. EVANS ET AL. 'MAPPING RECEPTOR BINDING SITES IN INTERLEUKIN (IL)-1 RECEPTOR-ANTAGONIST AND IL-1 BETA BY SITE-DIRECTED MUTAGENESIS.' cited in the application see figure 1A -----	1-13

1

INTERNATIONAL SEARCH REPORT

Info. ..ion on patent family members

International ..plication No
PCT/EP 95/03708

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9216221	01-10-92	AU-B- 1674292	21-10-92
		CA-A- 2106079	16-09-92
		EP-A- 0575545	29-12-93
		JP-T- 6506218	14-07-94
		NO-A- 933270	01-11-93